DECLARATION

I, Yuka INOUE of c/o The Patent Corporate Body ARUGA PATENT OFFICE, 3-6, Nihonbashiningyocho 1-chome, Chuo-ku, Tokyo 103-0013 Japan do solemnly and sincerely declare that I well understand both Japanese and English languages and that I believe the attached English version is a true and complete translation of the extract of the Japanese patent application No.2002-360769.

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[Document Name] Specification

[Title of the Invention]

Vaccine for Bacterial Cold-water Disease in Fish

[Claims]

[Claim 1] A vaccine against bacterial cold-water disease in fish, comprising inactivated cells of Flavobacterium psychrophilium in a logarithmic growth phase or effective components of the cells.

[Claim 2] A vaccine composition for bacterial cold-water disease in fish, containing inactivated cells of Flavobacterium psychrophilium in a logarithmic growth phase or components of the cells.

[Claim 3] A method for preventing bacterial cold-water disease in fish comprising administering an effective dosage of inactivated cells of Flavobacterium psychrophilium in a logarithmic growth phase or components of the cells.

[Detailed Description of the Invention]

[0001]

[Technical Field to Which the Invention Pertains]

The present invention relates to a vaccine against bacterial cold-water disease in fishes and a method for preventing bacterial cold-water disease in fish using the vaccine.

[0002]

[Background Art]

Bacterial cold-water disease is a disease occurring in salmon, trout, ayu (sweetfish) and crucian carp in low water temperature seasons. This disease, which attacks young fish in low water temperature seasons and has a high mortality, was originally discovered in trout in North America. While the mortality rate is 20 to 50%, another problem is that sequelae such as ulcers remain on the surface of the fish that have escaped death.

[0003]

Although therapy for and prevention of cold-water disease include raising the water temperature or oral administration of sodium sulfizole, raising the water temperature above 25°C is highly uneconomical while administration of drugs is not preferable for edible fish.

[0004]

It has been proved that the pathogen of the cold-water disease is Flavobacterium psychrophilium, which is also known as Flexibactor cyclophils or Cytophagar cyclophils. However, no vaccines against this disease have been developed.

[0005]

[Problems to be Solved by the Invention]

An object of the present invention is to provide a vaccine against bacterial cold-water disease in fish.

[0006]

[Means for Solving the Problems]

The inventors of the present invention have investigated Flavobacterium psychrophilium as a pathogen of the cold-water disease in terms of pathogenicity and vaccine activity depending on various cultivation conditions, and found a quite unexpected result in that the vaccine activity becomes higher by using bacteria in a logarithmic growth phase rather than using bacteria in a steady-state phase. The present invention has been completed through the discoveries above.

[0007]

The present invention provides a vaccine for bacterial cold-water disease in fish comprising inactivated cells of Flavobacterium psychrophilium in a logarithmic growth phase or components of the cells.

[0008]

Further, the present invention provides a vaccine composition for bacterial cold-water disease in fish containing inactivated cells of Flavobacterium psychrophilium in a logarithmic growth phase or components of the cells.

[0009]

Still further, the present invention provides a method for preventing bacterial cold-water disease in fish

comprising administering an effective dosage of inactivated cells of Flavobacterium psychrophilium in a logarithmic growth phase or components of the cells.

[0010]

[Means for Carrying Out the Invention]

Inactivated cells of Flavobacterium psychrophilium (may be referred to as the bacteria of the present invention hereinafter) in a logarithmic growth phase or components of the cells are used in the vaccine of the present invention. Usually, bacterial cultivation phases are divided into a lag phase, logarithmic growth phase, stationary phase, extinction phase and survival phase. Many projections were observed on the surface of invading bacterial cells upon observation of the bacterial cells of the present invention invading fish bodies. On the other hand, differences of cell secretory products were detected by SDS-PAGE and the existence of projections was observed on the surface of the bacterial cells in the logarithmic growth phase upon observation of the configuration and analysis of the bacteria of the present invention in the lag phase, logarithmic growth phase and stationary phase.

[0011]

The bacterial cells of the present invention used for production of the vaccine are obtained by cultivating the cells according to conventional methods and by harvesting

the cells in the logarithmic growth phase. The bacterial cells of the present invention may be seeded on an appropriate culture medium and cultivated according to conventional methods. The culture medium preferably contains an appropriate amount of assimilatable carbon and nitrogen sources.

[0012]

While the carbon and nitrogen sources are not particularly restricted, examples of them include tripton, serum of various animals, corn gluten meal, soy bean powder, corn steep liquor, casamino acid, yeast extract, pharma media, sardine meal, meat extract, peptone, HiPro®, AjiPower®, corn meal, soy bean meal, coffee refuse, cotton seed oil refuse, Cultivator®, Amiflex® and Ajipron®, Zest® and Ajix®. Examples of the carbon source include assimilatable carbon sources such as arabinose, xylose, glucose, mannose, sucrose, maltose, soluble starch, lactose and cane molasses, and assimilatable organic acids such as acetic acid. Phosphates, organic salts such as Mg^{2+}, Ca^{2+}, Mn^{2+}, Zn^{2+}, Co^{2+}, Na^{+} and K^{+} salts, and other inorganic salts and trace amounts of nutrients, if necessary, may also be added to the culture medium. Commercially available culture media such as TY culture medium and Cytophagar (CYT) culture medium, as well as modified Cytophaga (MCYT) culture medium and culture medium supplemented with bovine fetal serum may

also be used.

[0013]

The cultivation condition is preferably controlled at pH 6.8 to 8.4 and at a temperature of 4 to 20°C.

[0014]

Whether the bacteria of the present invention are in the logarithmic growth phase or not may be confirmed by measuring the optical density at 600 nm, which dramatically increases in the logarithmic growth phase. For example, cultivation reaches the logarithmic growth phase after 20 to 30 hours' cultivation at pH 7.3 and 15°C.

[0015]

The bacteria of the present invention in the logarithmic growth phase are separated by centrifugation or filtration, or the culture product may be directly inactivated. The inactivation treatment includes heat treatment or formalin treatment.

[0016]

The bacteria of the present invention contain cell membrane components, vesicles and secretary products. These components are preferably collected after ultrasonic pulverization of the inactivated bacterial cells.

[0017]

The inactivated bacterial cells and components thereof are preferably used after filtration, or after concentration

by evaporation or freeze drying.

[0018]

Although the inactivated bacterial cells of the present invention may be directly used as the vaccine, they may be formulated into a vaccine composition together with a pharmaceutically acceptable liquid or solid carrier. Examples of the formulation of the vaccine composition include oral administration compositions, injection compositions, compositions for immersing fish, and feed compositions. Examples of the liquid carrier include water and physiological saline, while examples of the solid carrier include excipients such as talc and sucrose. The inactivated bacterial cells of the present invention or components thereof may be mixed with conventional fish feeds to prepare the feed composition. An adjuvant may be added to these vaccine compositions in order to enhance the antigenicity.

[0019]

While the vaccine or vaccine composition of the present invention may be administered to adult fish, it is preferably administered before the onset of cold-water disease, for example, during the period when the fish is young. The dosage is preferably 1 mg to 5 g per 1 kg of the body weight as converted into the weight of the inactivated bacterial cells or components thereof. The dosage may be

once or several times, for example 2 to 10 times. The vaccine may be administered every day, or with an interval of 1 to 2 days.

[0020]

The fish that can be administered the vaccine or vaccine composition of the present invention are not particularly restricted so long as the fish are afflicted by cold-water disease caused by the bacteria of the present invention; examples of the fishes include ayu (sweetfish) and crucian carp, and salmon and trout such as yamame (salmo masau), rainbow trout and silver trout.

[0021]

[Examples]

While the present invention is described in more detail hereinafter with reference to examples, the present invention is by no means restricted to these examples.

[0022]

[Example 1]

(1) Cells of Flavobacterium psychrophilium G3724 (this strain was used in the experiments hereinafter) contained in a platinum loop were seeded on a 4-mL MCYT culture medium (trypton 2.0 g, yeast extract 0.5 g, meat extract 0.2 g, sodium acetate 0.2 g, calcium chloride 0.2 g, distilled water 1000 mL, pH 7.2). After cultivation at 15°C for 2 days, a 0.5-mL fraction of the culture medium was seeded on

a 200-mL MCYT culture medium followed by cultivation with shaking at 15°C. The relationship between the cultivation time, and the cell number and optical density at 600 nm is shown in Fig. 1. Fig. 1 shows that the lag phase is from 0 to 24 hours after seeding, the logarithmic growth phase is 24 to 48 hours after seeding, and the stationary phase is after 48 hours from seeding in the bacteria of the present invention.

[0023]

(2) The differences in pathogenicity of the bacteria of the present invention depending on the culture conditions were investigated. The bacteria of the present invention in the logarithmic growth phase and stationary phase were added to an aquarium of ayu at a concentration of 10^8 to 10^{10} CFU/mL to determine the pathogenicity of the bacteria. Ayu used for the experiment had a body weight of 0.5 to 5 g, and the temperature of the aquarium was 15°C. As shown in Fig. 2, while the mortality rate of the fish in the infection group using the bacteria of the present invention in the stationary phase until day 10 of the experiment was 20 to 60% of the mortality rate of the fish in the control group (non-infection group), the mortality of the fish in the infection group using the bacteria of the present invention in the logarithmic growth phase at day 10 of the experiment was 100%, showing that the bacteria in the logarithmic

growth phase have higher pathogenicity than the bacteria in the stationary phase.

[0024]

(3) The bacterial cells of the present invention in different growth phases were pulverized by ultrasonic waves. Each fraction of the extract was isolated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE, silver staining). The results are shown in Fig. 3. The results show that certain substances are specifically produced in the logarithmic growth phase (indicated by arrows in the graph).

[0025]

(4) The bacteria of the present invention in the logarithmic growth phase and stationary phase were observed under a scanning electron microscope (Fig. 4) and transmission electron microscope (Fig. 5). It was revealed from the results that projections can be seen on the surface of the bacterial cells in the logarithmic growth phase.

[0026]

(6) Ayu were infected with the bacteria of the present invention in the logarithmic growth phase. It was observed under the scanning electron microscope that the bacteria of the present invention had invaded into the lower jaw of the ayu (Fig. 6). The result indicates that the bacteria of the present invention in the logarithmic growth phase having the

vesicles invaded into the body of the ayu.

[0027]

[Example 2]

Flavobacterium psychrophilium G3724 was cultivated in 1000 mL of the MCYT culture medium contained in a 2000-mL Sakaguchi flask at 15°C. The cells showing OD 0.2 to 0.7 at 600 nm were used as the bacterial cells in the logarithmic growth phase. Then, the cells as a culture product at a growth phase showing OD of 0.2 to 0.7 at 600 nm in the culture period of 24 to 36 hours were inactivated by incubation in 0.3% formalin at 15°C for 2 days, and the inactivated bacterial cells were isolated by centrifugation at 4°C and 8,000 to 10,000 × g. The bacterial cells in the stationary phase after 36 hours' cultivation (OD_{600nm} = 1.0) were also inactivated by the same method as described above to obtain inactivated bacterial cells as controls.

[0028]

[Example 3]

Cells of Flavobacterium psychrophilium G3724 contained in a platinum loop was seeded on 50 mL of the MCYT culture medium and pre-cultivated at 15°C for 48 hours. A 2.5-mL fraction of this culture medium was seeded on 1000 mL of the MCYT culture medium, followed by cultivation at 15°C for 36 hours. OD at 600 nm was 02 to 0.7. The cultivation product was incubated in 0.3% formalin at 15°C for 2 days. The

bacterial cells were then collected by centrifugation at 8,000 to 10,000 × g at 4°C. The cells obtained were re-suspended in physiological saline containing 0.3% formalin to obtain a vaccine suspension containing the inactivated bacterial cells of the present invention.

[0029]

[Example 4]

The inactivated bacterial cells obtained from the cells in the logarithmic growth phase and stationary phase in Example 2 were orally administered to ayu with an average body weight of 5.0 g at a dosage of 0.1 FKCg/kg/day.

After the oral administration as described above, the ayu were challenged by immersing in the bacterial solution. The results are shown in Table 1.

[0030]

TABLE 1

Group	Dosage of Challenge	Death/ Challenge	Survival Rate
Logarithmic Growth Phase Group	1.7×10^8	39/152	$74^{a,c}$
Stationary Phase Group	1.9×10^8	39/105	63^{b}
Control Group	2.2×10^8	82/165	50

a: Significant difference against control group ($p < 0.001$), chi-square test

b: Significant difference against control group ($p < 0.05$)

c: Significant difference against control group ($p < 0.05$)

[0031]

Table 1 shows that the difference in the survival rate was significant in both the stationary phase group and logarithmic growth phase group as compared with the control group. However, the survival rate of the logarithmic growth phase group was significantly higher than that of the stationary phase group, showing that the logarithmic growth phase group is particularly useful as the vaccine.

[0032]

[Example 5]

The effect of the vaccine was investigated using the vaccine composition obtained in Example 3. The vaccine was orally administered for 2 weeks (0.1 g/kg) to the fish from 5 weeks before the start of challenge, and the fish were fed on a standard feed for 3 weeks to enhance immunological activity. The fish were then divided into two groups: one in which the challenge was started 3 weeks after the end of vaccine administration, and another in which the challenge was started 7 weeks after the end of vaccine administration.

Two thousand "ayu" with a body weight of 0.5 g were divided into two groups. The vaccine was either orally administered every day to the fish in one group, or five times in two weeks (oral administration with an interval of two days) to the fish in the other group. The results are shown in Table 2, and in Figs. 7 and 8.

[0033]

TABLE 2

	Average Body Weight (g)	Amount of Challenge (CFU/mL)	No. of Deaths/No. of Challenges	Survival Rate (%)
Challenge 1[a]				
1	1.7		7/118	94.1[b]
2	1.8	4.4×10^7	4/119	96.6[b]
Control	1.8		36/117	69.2
1	1.9		53/114	53.5
2	1.8	1.2×10^8	10/120	91.7
Control	1.9		79/121	34.7
Challenge 2[a]				
1	2.7		26/186	86.6[b]
2	2.9	2.1×10^7	20/168	88.1[b]
Control	2.7		41/174	76.4
1	2.7		40/170	76.5[b]
2	3.0	1.4×10^8	36/165	78.8[b]
Control	3.2		107/185	42.2

a: Challenge 1: challenged 3 weeks after administration of vaccine, Challenge 2: challenged 7 weeks after administration of vaccine

b: significant difference against control group ($p < 0.01$)

1: the group in which the vaccine was administered every day for 2 weeks

2: the group in which the vaccine was administered 5 times in 2 weeks

[0034]

The results of the challenge tests three weeks after

the administration of the vaccine show that a significant difference was observed between the vaccine-administered group and control group. It was also shown that the effect of the vaccine is higher in the group in which the vaccine was administered only five times than in the group in which the vaccine was administered every day.

The effect of the vaccine was significantly higher in both vaccine-administered groups than in the control group, when the challenge test was performed 7 weeks after administration of the vaccine.

[0035]

In the test fish that died in the test period of the present invention, it was confirmed whether the death was ascribed to the bacteria of the present invention or not. As shown in Figs. 9 and 10, typical symptoms of the cold-water disease were observed in all the dead fish. It was also revealed that the cause of death of the test fish during the test period of the present invention was infection with the bacteria of the present invention, since staining of the dead fish with a fluorescent antibody was positive with respect to all the individuals tested.

[0036]

[The Effect of the Invention]

Using the vaccine of the present invention can efficiently prevent bacterial cold-water disease of salmon,

trout, carp and ayu.

[Brief Description of the Drawings]

Fig. 1 is a graph showing the relationship between the cultivation time and optical density (OD) at 600 nm and the number of cells (CFU/mL);

Fig. 2 is a graph showing the pathogenicity (accumulated mortality) of ayu depending on the cultivation conditions of the bacteria of the present invention;

Fig. 3 shows a the results of the SDS-PAGE analysis of the cell components of the bacteria of the present invention;

Fig. 4 shows scanning electron microscope photographs (A, C and E = 20,000 times of magnification,; B, D and F = 100,000 times of magnification) of the bacteria of the present invention at logarithmic growth phases (A and B: 36 hours) and at stationary phases (C and D: 48 hours, E and F: 72 hours);

Fig. 5 shows transmission electron microscope photographs of ultra-thin slices of the bacteria of the present invention in the logarithmic growth phase.

Fig. 6 shows scanning electron microscope photographs of the lower jaw of ayu infected with the bacteria of the present invention;

Fig. 7 shows the survival rate in challenge 1

(challenge 3 weeks after administration of the vaccine);

Fig. 8 shows the survival rate in challenge 1 (challenge 7 weeks after administration of the vaccine);

Fig. 9 is a photograph showing the symptoms of the dead ayu (the arrows show the symptoms specific to the cold-water disease); and

Fig. 10 is a photograph showing the results of diagnosis of infection, if any, of dead ayu with the bacteria of the present invention detected using a fluorescent antibody.